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Mutual dependence of calcitonin-gene related peptide and acetylcholine release in neuromuscular preparations

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Abstract

To investigate the mutual dependence of calcitonin gene-related peptide (CGRP) and acetylcholine release, we examined the effect of a cholinesterase inhibitor neostigmine on the release of CGRP-like immunoreactivity in rat phrenic nerve-hemidiaphragm muscle preparation, and conversely, the effect of CGRP on [^{3}H]acetylcholine release from motor nerve terminals loaded with [^{3}H]choline in the same preparations of mice. Release of CGRP-like immunoreactivity was increased by electrical nerve stimulation (train of 40 pulses of 200 μs pulse duration and frequency of 50 Hz applied every 10 s) in the whole preparation but not in the segmental preparation containing the endplate region. Neostigmine (0.1–0.3 μM) enhanced the resting release of CGRP-like immunoreactivity in a concentration-dependent manner, whereas it depressed the nerve-evoked release of CGRP-like immunoreactivity. CGRP (1 μM) added to perfusate decreased nerve-evoked [^{3}H]acetylcholine release. These results suggest that CGRP, which is released by electrical nerve stimulation or a cholinesterase inhibitor in intact skeletal muscles, negatively modulates nerve-evoked acetylcholine release. © 1997 Elsevier Science B.V.

Keywords: CGRP (calcitonin gene-related peptide) release; Acetylcholine release; Neostigmine; Phrenic nerve-diaphragm muscle preparation

1. Introduction

Calcitonin gene-related peptide (CGRP) is a neuropeptide which is distributed widely in the central and peripheral nervous systems (Rosenfeld et al., 1983; Gibson et al., 1984). CGRP coexists with acetylcholine in motor nerve terminals (Takami et al., 1985b; Matteoli et al., 1988) as well as in sensory nerve terminals in skeletal muscle (Takami et al., 1985a; Ohlen et al., 1987). In the rat neuromuscular preparation, CGRP is released from skeletal muscle by electrical nerve stimulation with a long pulse duration (Uchida et al., 1990) or by high-frequency pulses (Csillik et al., 1993; Sala et al., 1995) and by application of high K^+ solution (Sakaguchi et al., 1991).

Endogenous CGRP promotes nicotinic acetylcholine receptor-operated Ca^{2+} mobilization and enhances desensitization of nicotinic receptors in the phrenic nerve-hemidiaphragm muscle preparation treated with cholinesterase inhibitors (Kimura et al., 1993). The Ca^{2+} mobilization is triggered by the prolonged action of acetylcholine, which has accumulated in the synaptic cleft as a result of inhibition of cholinesterase (Kimura et al., 1991a). These data suggest that the release of CGRP may be triggered by the action of acetylcholine accumulated at the synapse.

To investigate the mutual dependence of CGRP and acetylcholine release from skeletal muscle, we examined (1) the effect of neostigmine, which increases the availability of acetylcholine at the neuromuscular synapse, on the release of CGRP (determined by radioimmunoassay) in rat phrenic nerve-hemidiaphragm muscle preparations and conversely (2) the effect of CGRP on acetylcholine release in the same preparations of mice.

2. Materials and methods

2.1. Muscle preparations

Male Wistar rats (6–9 weeks old, 170–250 g) were lightly anaesthetized with diethyl ether, then decapitated

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and bled. Both right and left phrenic nerve-hemidiaphragm muscles were isolated and 'fan-shaped' preparations were obtained together with the attached rib segment (Wessler and Kilbinger, 1986). The rib end and the tendon of the preparation were pinned to rubber plates in a chamber. In some experiments a 2 to 3 mm wide strip containing the endplate region (segmental preparation) was used. These preparations were suspended in 3 ml Krebs solution containing 10 μg/ml aprotinin and 250 μM phenylmethylsulfonyl fluoride and gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Krebs solution was composed of (in mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.5.

2.2. Measurement of released CGRP by radioimmunoassay

The release of CGRP-like immunoreactivity was measured by radioimmunoassay (Uchida et al., 1990). After an equilibration period of 20 min, the preparation was incubated for 10 min in 3 ml Krebs solution and then the solution was collected into an assay tube for radioimmunoassay to measure resting CGRP release. The phrenic nerve was then stimulated electrically (train of 40 pulses of 10–10 000 μs duration and frequency of 50 Hz applied every 10 s, 2.5 V) for 10 min. Each sample solution in an assay tube was lyophilized and dissolved in 0.6 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 0.3% bovine serum albumin and 10 mM ethylenediaminetetraacetic acid. The solution was then incubated with rabbit antiserum against human CGRP and [^{125}I]CGRP for 5 days at 4°C. Synthetic rat CGRP was used as a standard for assay.

2.3. Measurement of released [^{3}H]acetylcholine

The effect of CGRP on acetylcholine release from motor nerve terminals was studied in the same 'fan-shaped' preparations from male ddY mice (7–10 weeks, 30–41 g). The release of acetylcholine evoked by electrical stimulation was measured by a radioisotope method, as previously reported (Kimura et al., 1991b). The preparation was incubated for 60 min in 3 ml Krebs solution containing methyl-[^{3}H]choline (185 kBq ml^{-1}). To facilitate the uptake of [^{3}H]choline into the acetylcholine pool in motor nerve terminals, the phrenic nerve was stimulated electrically for 40 min, with pulses of 200 μs duration. After a 20 min washout, the preparation was superfused with Krebs solution at a rate of 1 ml/min and electrical stimulation as described above was started at 8 (S_0), 32 (S_1) and 56 (S_2) min. Samples were collected at 1 min intervals from 6 min before the 32 min point (S_1). The effect of CGRP on the evoked release of acetylcholine was determined from changes in the S_2/S_1 ratios. The nerve-evoked increase in radioactivity was calculated by subtracting the mean of the basal release from that of the evoked release. The mean basal release was calculated from the values of 6-min fractions before and after a stimulation period. The radioactivity of the samples was measured in a scintillation beta spectrometer (LS 3801, Beckman, Fullerton, CA, USA).

2.4. Statistics

The statistical significance of the differences between the treated groups and the corresponding control was determined by one way analysis of variance followed by Scheffe's test. $P < 0.05$ was adopted as the level of significance.

2.5. Drugs

Synthetic α-CGRP (rat; Peptide Institute, Osaka, Japan), (2-[^{125}I]iodohistidyl^{10})CGRP (human, 74 TBq mmol^{-1}), rabbit antiserum against human CGRP for radioimmunoassay, methyl-[^{3}H]choline chloride (3.2 TBq mmol^{-1}) (Amersham, Amersham, UK), neostigmine methylsulphate, phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) and aprotinin (Bayer, Leverkusen, Germany) were used.

3. Results

3.1. Electrical nerve-stimulation evoked CGRP-like immunoreactivity release from skeletal muscle

We examined whether CGRP-like immunoreactivity is released from the isolated phrenic nerve-hemidiaphragm muscle by electrical stimulation with a short pulse duration. When the nerve was stimulated with a supramaximal voltage of 2.5 V (3–4 times the threshold for evoking muscle contraction), a pulse duration of 200 μs and trains of 40 pulses at 50 Hz applied every 10 s for 10 min to produce muscle contractions in whole preparation, the amount of CGRP-like immunoreactivity measured in the Krebs solution (23.2 ± 4.0 fmol/hemidiaphragm, $n = 6$; mean ± S.E.M.) increased significantly above the resting level (10.5 ± 2.8 fmol, $n = 6$) (Fig. 1, left columns). In the segmental preparation, in which muscle fibers were cut off so as to contain the endplate region, the resting level of CGRP-like immunoreactivity decreased significantly to a value of 2.2 ± 0.6 fmol ($n = 8$) compared with that in the whole preparation and the amount released by nerve stimulation did not increase at all (1.9 ± 0.5 fmol, $n = 8$) (Fig. 1, right columns). These results indicate that electrical nerve stimulation with a short pulse duration causes CGRP release in whole preparations of phrenic nerve-hemidiaphragm muscle, but not in segmental preparations.

3.2. Enhancing effect of acetylcholine accumulated at the neuromuscular synapse on resting release of CGRP-like immunoreactivity

To examine whether accumulated acetylcholine affects the release of CGRP, we investigated the effect of the

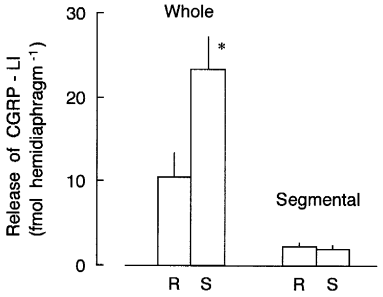

Fig. 1. Release of CGRP-like immunoreactivity (CGRP-LI) from whole (left) and segmental (right) preparations induced by electrical stimulation of the nerve (trains of 40 pulses of 200 μs duration and frequency of 50 Hz applied every 10 s for 10 min) in rat phrenic nerve-hemidiaphragm muscle preparations. The tissues were incubated for 20 min after equilibration. The incubation media for the first 10 min was used to measure resting release (R). In the second 10 min, the nerve was stimulated electrically (S). Significant differences between resting release and electrically evoked release were analyzed by a paired *t*-test (* $P < 0.05$). Values are means ± S.E.M. of 6 (whole) and 8 (segmental) experiments.

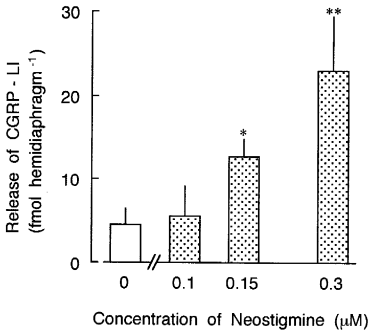

Fig. 2. Increasing effect of neostigmine on resting release of CGRP-like immunoreactivity (CGRP-LI) from rat phrenic nerve-hemidiaphragm muscle preparations. The tissues were incubated for 10 min with (0.1, 0.15 and 0.3 μM; stippled columns) or without (open column) neostigmine after equilibration. Significant differences from the control (without drug) were analyzed by one-way analysis of variance followed by Scheffe's test (* $P < 0.05$ and ** $P < 0.01$). Values are means ± S.E.M. of 3 to 7 experiments.

cholinesterase inhibitor neostigmine (0.1–0.3 μM) on CGRP release in whole preparations of rat hemidiaphragm muscle. Bath-applied neostigmine increased the resting release of CGRP in a concentration-dependent manner and significantly at doses of 0.15 and 0.3 μM (12.8 ± 2.1 fmol/hemidiaphragm; $n = 3$ and 22.9 ± 6.5 fmol; $n = 7$, respectively), compared with that in the absence of neostigmine (4.4 ± 2.0 fmol; $n = 5$) (Fig. 2).

3.3. CGRP-like immunoreactivity release from rat phrenic nerve-hemidiaphragm muscle by electrical nerve stimulation at various pulse durations and depressant effect of neostigmine

We investigated the dependence of nerve-evoked CGRP-like immunoreactivity release from rat skeletal muscle on the pulse duration of electrical nerve stimulation. The CGRP-like immunoreactivity released in the presence of 0.3 μM neostigmine was measured simultaneously. The pulse duration of electrical stimulation was changed from 10 to 10 000 μs. The release of CGRP-like immunoreactivity tended to increase with prolongation of pulse duration (above 10 μs) and it reached a maximum at 100 μs duration (Fig. 3). In the presence of 0.3 μM neostigmine, the nerve-evoked CGRP-like immunoreactivity release was depressed significantly over a range of pulse durations of 100 μs to 1000 μs.

3.4. Depressant effect of CGRP on acetylcholine release from motor nerve

The effect of CGRP on tritium overflow from the mouse phrenic nerve-hemidiaphragm muscle preparations was examined. Fig. 4a shows the tritium overflow after the washout period in the absence and presence of 1 μM CGRP. Electrical nerve-evoked tritium release from the preparations loaded with [^{3}H]choline was induced by two consecutive electrical stimulation periods (S_1 and S_2). The electrical nerve-evoked increase in tritium release over the level of spontaneous output can be attributed to [^{3}H]acetylcholine release from the nerve terminal, as shown in our previous paper (Kimura et al., 1993). The concentration–response curve of CGRP for acetylcholine release (S_2/S_1) is shown in Fig. 4b. The value of S_2/S_1 in the presence of 1 μM CGRP (0.53 ± 0.07, $n = 4$; mean ± S.E.M.) was significantly smaller than the value in the absence of CGRP (0.76 ± 0.02, n = 4). These data indicate

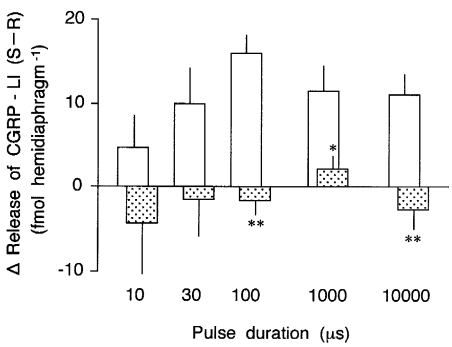

Fig. 3. The dependence of CGRP-like immunoreactivity (CGRP-LI) release from rat phrenic nerve-hemidiaphragm muscle preparations on the pulse duration of electrical nerve stimulation and its depression by 0.3 μM neostigmine. The tissues were incubated for 20 min with (stippled columns) or without (open columns) neostigmine after equilibration. Data indicate electrically-evoked CGRP-LI for 10 min calculated by subtracting resting release in the first 10 min from the total release with electrical stimulation (10 to 10 000 μs duration) in the second 10 min. Significant differences from the control (without neostigmine) were analyzed by one-way analysis of variance followed by Scheffe's test (* $P < 0.05$ and ** $P < 0.01$). Values are means ± S.E.M. of 4 to 8 experiments.

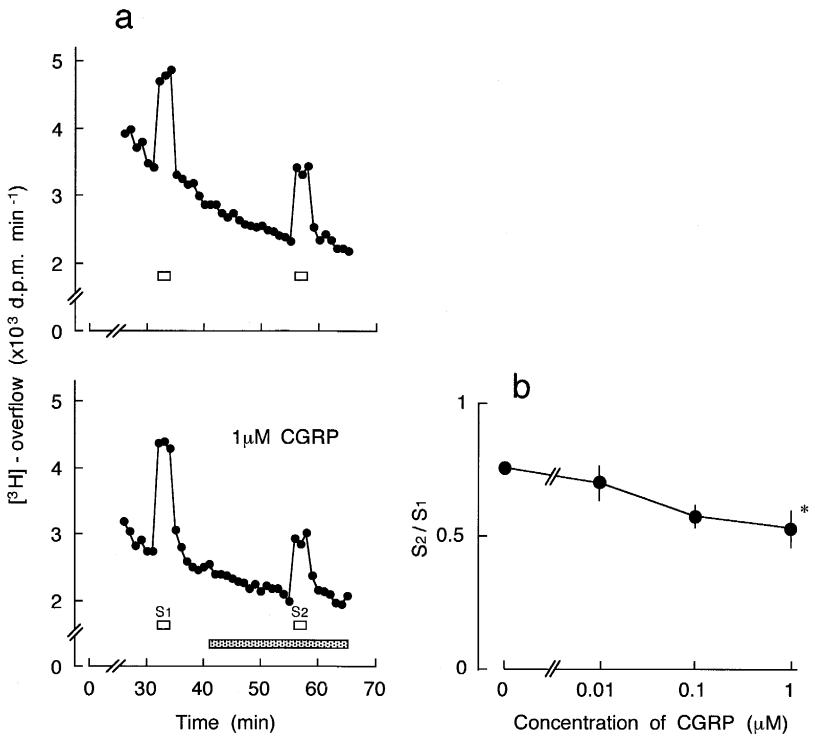

Fig. 4. The effects of bath-applied CGRP on nerve stimulation evoked [^{3}H]acetylcholine release from mouse phrenic nerve-hemidiaphragm muscle preparations. (a) Time-course of tritium overflow (upper, control; lower, CGRP 1 μM). [^{3}H]Acetylcholine release was elicited by two stimulation periods (S_1 and S_2), as indicated by the open columns. The stippled column indicates the presence of CGRP. Each point represents the mean value ($n = 3$). The horizontal axis indicates the time after the end of the washout period. (b) Concentration–response curve of CGRP for electrically evoked [^{3}H]acetylcholine release (S_2/S_1) from phrenic nerve-diaphragm muscle preparations. CGRP was applied in various concentrations 15 min before S_2 as indicated in (a). Each point represents the mean value for relative ratios of acetylcholine release (S_2/S_1) ($n = 3$–5) and vertical bars represent S.E.M. Significant differences from the control (without drug) were analyzed by one-way analysis of variance followed by Scheffe's test (* $P < 0.05$).

that CGRP (1 μM) depressed electrical stimulation-evoked [^{3}H]acetylcholine release from the skeletal muscles.

4. Discussion

CGRP, which coexists with acetylcholine, is a slow-acting trophic factor in the regulation of postsynaptic activity (Changeux et al., 1992) and regulates the number (Fontaine et al., 1986; New and Mudge, 1986) and the function (Mulle et al., 1988) of nicotinic acetylcholine receptors at the developing neuromuscular synapse. CGRP modulates postsynaptic activity in the mature neuromuscular synapse. Electrical nerve stimulation with a long pulse duration of 10 ms increases the cyclic AMP content of rat hemidiaphragm muscles, an effect which is abolished by pretreatment with antiserum against CGRP (Uchida et al., 1990). We have demonstrated that the extent of nerve-evoked intramuscular Ca^{2+} mobilization, which enhances desensitization of nicotinic receptors, is diminished by administration of a CGRP receptor antagonist CGRP-(8–37) in mouse phrenic nerve-diaphragm muscle preparations (Kimura et al., 1993). These data suggest that CGRP could be released from skeletal muscle preparations by electrical nerve stimulation.

In the whole muscle preparation, CGRP was released by electrical nerve stimulation with a pulse of 200 μs duration, which is considerably shorter than that reported by Uchida et al. (1990). This result suggests that CGRP may be released from motor nerve terminals in skeletal muscles during tetanic contraction, though the major source of CGRP released in skeletal muscles by electrical nerve stimulation may include Aδ- and/or C sensory terminals (Sakaguchi et al., 1991). In the segmental preparation, electrical nerve stimulation failed to produce the release of CGRP. A prolonged depolarization either of intramuscular afferent nerves or of the muscle membrane itself may have induced CGRP release. The basal CGRP release was profoundly reduced in the segmental preparation when compared to that of the whole muscle preparation. This difference suggests that most CGRP released originates from intramuscular afferent nerves or muscle fibers. Therefore, CGRP may originate not only from phrenic nerve terminals but also mainly from intramuscular afferent nerves or/and from muscle fibers.

The release of CGRP is facilitated by nerve stimulation of high frequency in skeletal muscles of adult rats (Csillik et al., 1993; Sala et al., 1995). Exogenously applied acetylcholine increases the amount of CGRP released from rat trachea, via activation of nicotinic acetylcholine receptors

in the peripheral terminals of primary afferent nerves (Jinno et al., 1994). In this study, we found that neostigmine increased the resting release of CGRP in skeletal muscles and that a longer pulse duration was needed for the maximal evoked release of CGRP. These data indicate that prolonged muscle contraction, as induced by treatment with a cholinesterase inhibitor, may contribute to CGRP release. Electrical nerve stimulation in the presence of neostigmine did not increase the amount of CGRP released above the resting level. This may be due to the depression of muscle contraction resulting from desensitization of nicotinic acetylcholine receptors, because of the abnormal increase in acetylcholine.

In the present study, CGRP in the perfusate decreased electrically stimulated [^{3}H]acetylcholine release from mouse diaphragm muscles. CGRP reduces the quantal content of acetylcholine release at the frog neuromuscular junction (Caratsch and Eusebi, 1990). Correia-de-Sa and Ribeiro (1994) have reported that CGRP (0.2–0.8 μM) facilitates [^{3}H]acetylcholine release from rat motor nerve terminals loaded with [^{3}H]choline, which is different from our finding. They used segmental muscle preparations, where CGRP increased [^{3}H]acetylcholine release upon electrical stimulation with pulses of not 40 μs, but 1 ms duration. Adenosine is released from both nerve terminals and muscle fibers on electrical stimulation, and inhibits acetylcholine release from motor nerve terminals (Smith and Lu, 1991; Redman and Silinsky, 1994). In non-contractible segmental muscle preparations, adenosine may no longer be released from skeletal muscle. In contractible whole muscle preparations (the present study), CGRP may depress the electrical stimulation-evoked acetylcholine release from motor nerve terminals through the action of adenosine, which is increased during muscle contraction. CGRP enhances muscle contraction (Takami et al., 1985b). Therefore, CGRP may act as a first-acting depressant regulator to prevent an excessive action of acetylcholine at the neuromuscular synapse.

In conclusion, CGRP is released by electrical nerve stimulation or by a cholinesterase inhibitor in intact skeletal muscles and the CGRP may negatively modulate nerve stimulation evoked acetylcholine release.

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